IMMUNOGENICITY OF SODIUM DODECYL SULFATE-DENATURED PLANT VIRUSES AND PLANT VIRAL INCLUSIONS

By

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Seven plant viruses (clover yellow mosaic, papaya mosaic, pepper mottle, potato Y, potato X, southern bean mosaic, and tobacco etch) and the inclusions induced by pepper mottle and tobacco etch viruses were denatured in sodium dodecyl sulfate (SDS). The denatured viruses and inclusions were separated from free SDS by column chromatography, concentrated, and injected into rabbits. Immunogenicity of the injected preparations was demonstrated by agar gel immunodiffusion tests and specificities of the antisera were tested against homologous and heterologous antigens. Use of SDS-denatured plant viruses and inclusions as immunogens elicited antisera having titers and specificities comparable to antisera obtained by the methods of others.

INTRODUCTION

The use of agar gel immunodiffusion techniques to study many of the anisometric plant viruses requires the use of denaturants to disrupt (depolymerize) the intact virus particle into freely diffusible, antigenic fragments. Various denaturants have been used for this purpose: ethanolamine (Purcifull and Shepherd, 1964; Purcifull, 1966; Purcifull and Gooding, 1970), pyridine (Shepard, 1970), pyrrolidine (Shepard et al., 1971; Shepard et al., 1974), and the anionic detergents leonil (Hamilton, 1964; Shepard and Grogan, 1967) and sodium dodecyl sulfate (Gooding and Bing, 1970).

Sodium dodecyl sulfate (SDS), because of its ability to denature a wide variety of proteins (Steinhardt and Reynolds, 1970), appears to have considerable promise for general use with plant virus antigens. In addition to its use for viruses in the potato Y group (Gooding and Bing, 1970), it also has been used successfully to disrupt plant virus inclusions into diffusible antigenic fragments suitable for immunodiffusion analyses (Hiebert et al., 1971; Purcifull et al., 1973).

Various problems have arisen in the limited attempts to use SDS-denatured plant virus antigens in agar gel

immunodiffusion tests, and detailed information is lacking regarding the use of SDS for this purpose. In some instances, SDS-treated antigens were not immunoreactive (Stevenson and Hagedorn, 1973); in others they produced spurious, nonspecific precipitates (Uyemoto et al., 1972). The nonspecific precipitates that SDS also gives with serum components could be confused with specific immunoprecipitates (Palmer et al., 1971). In all the previous attempts to use SDS-treated plant virus antigens for serological tests, the antisera used were prepared either against untreated virus or inclusion preparations (Gooding and Bing, 1970; Purcifull et al., 1973) or against virus preparations denatured with chemicals other than SDS (Stevenson and Hagedorn, 1973; Shepard et al., 1974). The specificity and potency of antisera to potato virus X and its pyridine-denatured proteins are markedly influenced by the condition (intact or depolymerized) of the immunogen (Shepard and Shalla, 1970). By analogy, the potency and specificity of antisera to SDS-denatured antigens could be similarly affected.

The research reported herein was undertaken to test the applicability of SDS denaturation for a variety of viral antigens, to improve and delimit the SDS-immunodiffusion system, and to develop antisera to SDS-denatured immunogens. SDS was used to denature (1) several anisometric viruses,

including three in the potato X group and three in the potato Y group (Harrison et al., 1971); (2) cytoplasmic "pinwheel" inclusions induced by two viruses in the potato Y group (Edwardson, 1966; Hiebert et al., 1971); (3) nuclear inclusions induced by tobacco etch virus; and (4) one isometric virus (southern bean mosaic virus). The idea of using denatured viruses and virus-induced inclusions as test antigens and immunogens was critically evaluated. SDS was compared with other denaturants and its merits considered with regard to its use in agar gel immunodiffusion systems utilizing both purified antigens and antigens derived from the crude sap of infected plants. Evidence is presented to demonstrate the immunogenic nature of SDS-denatured antigens. It is also shown that the SDS-gel immunodiffusion system compares favorably with other gel immunodiffusion systems in which chemical denaturation (e.g., with leonil, pyrrolidine, or ethanolamine) of the test antigen is required for diffusion in agar gel media.

MATERIALS AND METHODS

Source of Viruses and Virus Purification

A mottle strain of potato virus X (PVX) obtained from J. F. Shepard was propagated in Nicotiana tabacum L. 'Samsun NN'. Clover yellow mosaic virus (CYMV), an isolate supplied by D. E. Purcifull, was propagated in pea (Pisum sativum L. 'Little Marvel'). Papaya mosaic virus (PMV) obtained from E. Hiebert was cultured in Papaya carica L. Southern bean mosaic virus (SBMV) provided by J. G. McDonald was propagated in Phaselous vulgaris L. 'Topcrop'.

All these viruses were purified from systemically infected host-plant tissue collected 3-10 wk after mechanical inoculation. Purification (Figure 1) was performed using a modification of a previously described method which featured chloroform-butanol clarification (Steere, 1956). The concentration of purified viruses was determined spectrophotometrically using extinction coefficients reported for each virus as follows: CYMV (Purcifull and Shepherd, 1964), PVX (Paul, 1959), PMV (Hiebert, 1970), SBMV (Ghabrial et al., 1967).

1. Systemically infected tissue (500 g) was harvested 3-10 weeks after inoculation and cooled to 4 C before use.

2. Tissue was homogenized in a blender with the following solution: 250 ml of 0.05 M sodium borate buffer, pH 8.2, containing 0.1% mercaptoethanol; 125 ml of n-butanol and 125 ml of chloroform. All items were cooled to 4 C before use.

3. The tissue slurry was centrifuged at 8,500 RPM for 10 min in a Sorvall, GSA rotor, using the refrigerated RC-2B Sorvall centrifuge.

4. The supernatant solution was poured through Pyrex glass wool into a cold, graduated cylinder. After noting the volume, the solution was poured into a beaker and refrigerated. Polyethylene glycol (M.W. 6000) was added (3%, w/v, for PVX, PMV, CYMV; 10% for SBMV) to the supernatant solution.

5. The PEG-supernatant solution was stirred for 1-2 hr. The precipitate was collected by centrifugation at 8,500 RPM for 20 min using the Sorvall, GSA rotor.

6. Supernatant solutions were discarded and pellets were resuspended in a total of about 100 ml of 0.02 M sodium borate buffer containing 0.01% mercaptoethanol. The resuspended material was collected and stirred overnight at 4 C.

7. The preparations were further purified and concentrated by at least two cycles of differential centrifugation. The final virus pellets were resuspended in 0.01 M sodium borate, pH 8.2, and the preparations stored at 4 C.

Figure 1. Purification procedure for PVX, PMV, CYMV, and SBMV.

All viruses were subjected to sucrose density gradient centrifugation prior to being used as immunogens. Linear 10-40% gradients were used for the rod-shaped viruses, while linear-log gradients (Brakke and van Pelt, 1970) were used for SBMV. Sucrose was prepared as a stock 60% solution in deionized water. Gradients were prepared from the stock sucrose using deionized water and 0.04 M sodium borate, pH 8.0, as diluents, to yield the desired sucrose concentration, and a final buffer concentration of 0.02 M. Gradients were refrigerated for 16-24 hr before their use. Linear gradients were centrifuged at 23,000 RPM for 2.5 hr using the Beckman Model L ultracentrifuge and the SW 25.1 swinging bucket rotor. Linear-log gradients were centrifuged at 39,000 RPM for 3 hr using the Beckman SW 41 swinging bucket rotor in a Model L2-65B Beckman ultracentrifuge. Gradients were analyzed using an Isco (Instrument Specialties, Co., Lincoln, Neb.) Model 640 density gradient fractionator coupled to a Model UA-4 ultraviolet analyzer and a Model 610 strip chart recorder.

Purified preparations (Hiebert and McDonald, 1973) of the following viruses and virus inclusions were kindly supplied by E. Hiebert: pepper mottle virus (PeMV), PeMV cytoplasmic inclusions (PeMVI), potato virus Y (PVY), tobacco etch virus (TEV), TEV cytoplasmic inclusions (TEVI), and TEV nuclear inclusions (TEVNI). Concentrations of purified viruses were

determined by means of extinction coefficients previously reported as follows: TEV (Purcifull, 1966), PVY and PeMV (Shepard et al., 1974). For quantitation of the inclusions, 0.1 ml of the purified preparation was diluted to 1 ml with deionized water containing 1% SDS. The solution was heated for one min in a boiling water bath and then centrifuged for 10 min at 2600 g to remove insoluble material. The supernatant's ultraviolet absorbency at 280 nm was recorded.

Immunogen Preparation

Denaturation by Sodium Dodecyl Sulfate

Viruses and viral inclusions for use as immunogens were denatured by sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (MCE). SDS (Sigma) was recrystallized once from hot ethanol, and MCE (Eastman) was used as received. To 1 ml of a viral or viral inclusion preparation, SDS and MCE were each added to give final concentrations of 3% (w/v and v/v, respectively). The virus preparations were used at concentrations of 2-4 mg/ml, and the inclusion preparations were adjusted to 3-5 absorbence units/ml at 280 nm. The solutions were placed in a boiling water bath for 1-2 min to insure complete denaturation. After heat treatment, the inclusion preparations were centrifuged at 4500 RPM for 15 min in a Sorvall Model SP

table centrifuge to remove insoluble material. 1.0 ml was layered onto a K15/30 Sephadex column packed with G-50-150 Sephadex gel. The samples were eluted by gravity flow using 0.02 M sodium borate, pH 8.0. A flow rate of 1-2 ml/min was employed, and the effluent was monitored as described for liquid column chromatography. Column calibration was performed using intact PMV (1 mg/ml) or TMV (1 mg/ml) as the exclusion volume (V_o) marker and MCE (1%) as the total inclusion volume (V_t) marker.

The peak appearing at the column V_o was collected in a 12 ml syringe fitted with a 400 mesh nylon net at the tip, which was sealed at the tip with Parafilm. Sephadex G-25-150 was added to the syringe and allowed to swell for 10-15 min. The seal covering the syringe tip was removed and the syringe was then centrifuged at 1500 RPM for 15 min in the Sorvall Model SP table centrifuge. The liquid was collected and the procedure was repeated until the desired volume was obtained (Technical Data Sheet-Solute Concentrating with Sephadex, Pharmacia Fine Chemicals).

A detailed spectrophotometric analysis of the various steps was performed using PMV. Denatured, concentrated preparations of TEV, PMV, and TEVI also were examined by electron microscopy. A solution of sodium phosphotungstate (2%, w/v, pH 6.8) and 0.1% bovine serum albumin was the negative staining

agent. It was mixed, drop for drop, with the concentrated denatured virus preparations and then placed onto an electron microscope grid, dried and observed in a Philips electron microscope.

Denaturation of PMV by Guanidine·HCl

PMV was the only immunogen subjected to guanidine·HCl denaturation. The guanidine (Mann Ultrapure) was prepared as a 5 M solution in deionized water. The denaturant was added 1:1 (v/v) to the PMV solution (5 mg/ml) and incubated, with occasional shaking, in an ice bath. After 2 hr, insoluble material and undenatured virus were removed by centrifugation (38,000 RPM, 1.5 hr, Beckman #40 rotor). The supernatant fraction was removed and dialyzed overnight against 4 M urea in 0.02 M sodium borate, pH 8.0. The preparation was then dialyzed for 16-18 hr against 0.02 M sodium borate, pH 8.0 containing 4 M urea and 0.05% formaldehyde (prepared from paraformaldehyde). A final dialysis was performed for 16-18 hr against the borate buffer. The preparation was removed from the dialysis membrane and centrifuged (Sorvall, SS-34 rotor, 10,000 RPM, 15 min) to remove any insoluble material. The resulting preparation was quantitated using an extinction coefficient ($E\ \frac{1\%}{1cm}$) of 12 at 280 nm.

Formaldehyde-Fixed
Immunogens

As previously described, guanidine·HCl-denatured PMV was formaldehyde fixed. In addition, denatured PMV and PVX were formaldehyde treated immediately after the removal of the denaturant solution (SDS-MCE) by Sephadex gel filtration. Fixation was accomplished by the direct addition of a concentrated, freshly prepared formaldehyde solution to the collected peak to give a final concentration of 0.5% (v/v) formaldehyde. The solutions were incubated for 2 hr, transferred to a dialysis membrane, and dialyzed overnight against 0.03% formaldehyde in 0.02 M sodium borate, pH 8.0. Residual formaldehyde was removed by overnight dialysis against 0.02 M sodium borate, pH 8.0 and the preparations were concentrated by G-25 Sephadex centrifugation. The PMV preparation was examined by SDS-polyacrylamide slab gel electrophoresis (Hiebert and McDonald, 1973).

Preparation and Processing of
Immune Sera

The depolymerized, viral immunogens were quantitated spectrophotometrically using the extinction coefficients for the intact, undenatured viruses. For injection, the solutions were adjusted to concentrations of 2-3 mg/ml in a total volume

of 1 ml or less. The denatured viral inclusions were handled as for the viruses except that their concentrations were adjusted to give 1-3 absorbence units per ml at 280 nm in a total volume of 1 ml or less. All immunogens were emulsified 1:1 with Freund's complete adjuvant (Difco) and injected intramuscularly into the hind legs of New Zealand white rabbits weighing 2-3 kg. In addition, intact PMV, SBMV, and SDS-denatured PMV were injected intramuscularly using 2-3 mg (in a volume of 1 ml) emulsified 1:1 with Freund's incomplete adjuvant (Difco). In all cases, a second injection was administered 30-40 days after the initial injection.

Sera collection was commenced 1-2 wks after the final injection. Whole blood from the marginal ear vein of the rabbit was collected in 30 ml glass centrifuge tubes. The tubes were kept in a water bath at 37 C for 1 hr, after which they were centrifuged for 10 min at 2000 RPM in a Model SP, Sorvall table centrifuge. The serum was drained from the clots directly into conical bottomed centrifuge tubes and these were centrifuged for 15 min at 4000 to 4500 RPM in the table centrifuge. The clear supernatant fluid from the second centrifugation was separated from the pellets into storage containers and immediately frozen. No preservatives were added.

Titering of Antisera

All antisera were titered using purified, homologous antigens. Antiserum to intact SBMV was titered in the 0.05 M sodium borate buffered immunodiffusion medium. Purified antigens were used at concentrations of 1 mg/ml (viruses) and 1 absorbence unit/ml at 280 nm (viral-induced inclusions). The antisera were serially, two-fold diluted using 0.01 M sodium borate, pH 8.0. Table 2 (in Results) details the serum collection dates titered and the nomenclature used to describe the various antisera.

For comparisons, PeMVSC, TEVSC, PVXSC, PVXSCF, PMVSC, and PMVSCF antisera also were titered, using purified viral antigens denatured in 3% pyrrolidine. The antigen concentration and serum dilutions were as described for titer determination in the SDS system. The agar gel immunodiffusion media and patterns were as described in the section on the Immunodiffusion Media and Gel Patterns.

Preparation of Antigens in Crude Tissue Extracts

Test antigens in crude sap extracted from virus-infected plants were used in many of the immunodiffusion experiments. One method of antigen preparation involved triturating tissue in deionized water (1 g/2 ml) and expressing the sap through

cheesecloth. The expressed sap was centrifuged for 30 min at 2,600 g, the supernatant fraction was discarded, and the pellet was resuspended in the desired denaturant (0.5 ml/g tissue) (Purcifull and Gooding, 1970). Another method for preparation of antigens in fresh tissue extracts (Purcifull et al., 1973) was used only when SDS was the denaturant. The procedure consisted of trituration of leaf tissue in water (1 ml/g tissue) followed by the addition of 1 ml of 3% SDS for each gram of tissue. The triturate was expressed through cheesecloth and added, without further treatment, directly to reactant depots.

Two other methods of crude sap antigen preparation involved lyophilization of the tissue sap extract. Fresh tissue was triturated either in deionized water or in 1.5% SDS (1 g tissue/2 ml). The sap was then expressed through cheesecloth, distributed into vials, and lyophilized. The lyophilized material was restored to the original volume before its use in immunodiffusion tests. Those samples lyophilized in SDS were resuspended with deionized water, and those lyophilized in water were resuspended in a selected denaturant such as ethanolamine (3%), pyrrolidine (3%), or SDS (1%). Sap extracts, lyophilized in water, were always used in tests involving other denaturants. Healthy tissue sap extracts were prepared as were those from diseased tissue and were used as controls in all tests.

Immunodiffusion Media and Gel Patterns

The immunodiffusion medium used for testing SDS-denatured antigens consisted of 0.8% Noble agar (Difco), 1% sodium azide (Eastmen, practical grade), and 0.5% SDS (all w/v) (Gooding and Bing, 1970). The agar was dissolved in deionized water and autoclaved to achieve full solution. After autoclaving, the agar was cooled to 60-65 C and the SDS and sodium azide were added. The medium was then distributed in 15 x 100 mm plastic petri plates (12 ml/plate). For testing of antigens denatured by reagents other than SDS, an immunodiffusion medium consisting of 0.6% Noble agar, 0.03% sodium azide, and 0.05 M sodium borate, pH 8.0 was used. The agar was added to the borate buffer and dissolved in a boiling water bath. After cooling to 60-65 C, the sodium azide was added and the medium was distributed into 15 x 100 mm plastic petri plates (10 ml/plate). All plates were stored in a humid chamber at 4 C. Other agar gel immunodiffusion media are described in the section on Results.

The patterns used in immunodiffusion were cut with an adjustable gel punch (Grafar Corp., Detroit, Mich.). The pattern most commonly consisted of a central well and six peripheral wells (all 7 mm in diameter) with the edge of the center well 5 mm from the edges of the peripheral wells. Two types of diffusion

patterns were used for titering. One was a trough pattern consisting of three circular wells (7 mm diameter) set on each side of and parallel to a 2 mm wide trough. The wells (antiserum) were spaced 5 mm from the edge of the trough (antigen). The other pattern consisted of three wells (1 antiserum and 2 antigen) 7 mm in diameter with the edge of the antiserum well 5 mm from the nearest edge of the antigen wells. For all immunodiffusion tests conducted, the plates were punched just prior to use and after filling the depots, the plates were placed into a moist chamber and incubated at 25 C.

Liquid Column Chromatography

Liquid column chromatography using controlled pore glass (CPG) was used to investigate the effects of various denaturants on viruses and inclusions. The CPG filtration medium is chemically inert and it was possible to use eluant systems containing denaturants that would normally destroy or significantly alter commonly used gel filtration matrices. Three eluant systems were used: 0.1% SDS in 0.02 M sodium borate, pH 8.0 (Collins and Haller, 1973); ethanolamine (3%, v/v) in deionized water; and pyrrolidine (3%, v/v) in deionized water.

A 1.2 x 109 cm glass column (Chromatronix, Berkeley, Ca.) fitted with flow adapters was packed with a wet slurry of 370

Angstrom CPG of 120/200 mesh (Haller, 1965). Samples were introduced onto the column by means of a Chromatronix sample injector Model SV-8031 fitted with a 0.5 ml sample loop. Elution was performed by means of an Isco Model 312 metering pump and the effluent was monitored by an Isco absorbence monitor and optical unit Model UA-4. The optical unit was fitted with a 1 cm flow cell and a Model 610 Isco external strip chart recorder was used to continuously record the chromatographic events.

Column calibration was performed with deionized water as the eluant. Tobacco mosaic virus was used as the column exclusion volume marker (Vo) and MCE was the total inclusion volume marker (Vt). Flow rates in most of the chromatographic runs were less than 2 ml per min. Commonly, a wavelength of 254 nm was selected for effluent monitoring, although 280 nm was sometimes used. When the highly alkaline reagents (ethanolamine and pyrrolidine) were used as eluants, the CPG column packing was flushed with deionized water immediately after the chromatographic run(s) until the effluent attained a neutral pH.

RESULTS

SDS Denaturation of Immunogens

Immunogen preparation following treatment with SDS-MCE was a rapid and simple procedure. The column chromatography not only removed all the free SDS but simultaneously removed the MCE, which, due to its ultraviolet absorbency, would have interfered with immunogen quantitation. Based on UV spectrophotometry, usually 90% or more of the preparations applied to the column were recovered in the final concentrated immunogen sample. The column chromatography step always yielded two peaks (Figure 2). Peak A, which eluted at the column's Vo, contained the denatured antigen (RNA and capsid protein with the virus preparations and protein with the inclusion preparations). Peak B, which eluted at the column's Vt, was composed of free SDS and MCE. Using the colorimetric procedure of Reynolds and Tanford (1970a), the amount of SDS recovered in peak B was, in general, 85-90% of that originally present. The remainder of the SDS was presumably bound to the protein and tests for SDS in peak A generally demonstrated the presence of from 10-15% SDS. The total amount of SDS

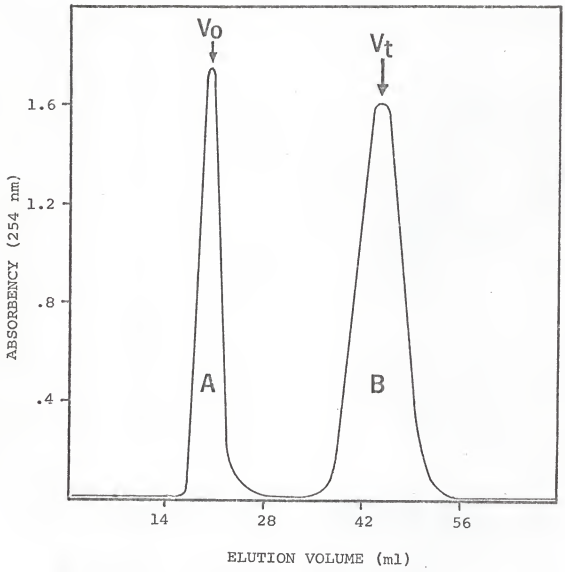


Figure 2. Illustration of SDS-MCE removal from SDS-denatured PMV by column chromatography using Sephadex G-50-150. Column- Sephadex K15/30; eluant- 0.02 M sodium borate, pH, 8.0.
(A), SDS-denatured antigen; (B), free SDS and MCE; (Vo), exclusion volume; (Vt), total inclusion volume.

detected did not necessarily correspond to that concentration of SDS originally added to the immunogen solution. This was assumed to be due to the detrimental effect on SDS of heating the sample in the boiling water bath.

The immunogen solution (peak A) was concentrated by G-25 Sephadex immediately after collection. The volume of peak A was normally about 10 ml and usually required a four-step procedure to achieve the desired final volume, approximately 1 ml. The denatured immunogen precipitated from solution in the absence of free SDS and the rate of precipitate formation was markedly accelerated after G-25 Sephadex concentration. Therefore, processing of the immunogen was done as rapidly as possible.

The denatured viruses were quantitated by means of the viral extinction coefficients. Because the procedure for preparation of the immunogen did not separate the viral RNA and protein, the UV absorption spectra of the denatured and intact virus preparations were very similar. The congruency was typical of the viruses used in this study. For example, Figure 3 shows the absorption spectra of PMV at two stages of immunogen preparation and compares them with the absorption spectrum of the intact virions.

The denaturation of viruses and viral-induced inclusions by SDS-MCE was very effective for most antigens. Electron

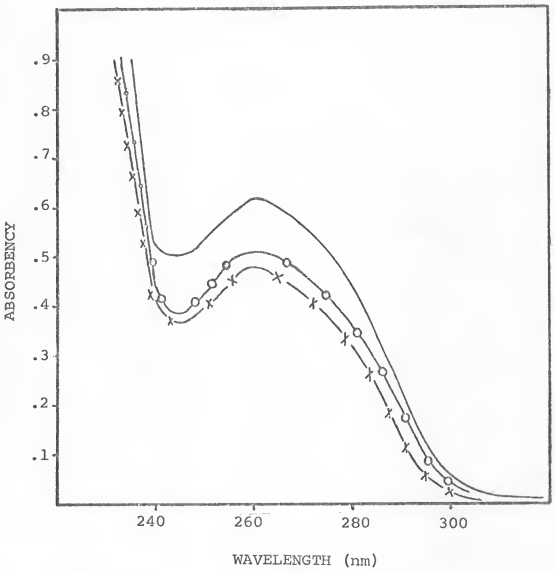


Figure 3. Absorption spectra of PMV at three stages of preparation of the SDS-denatured immunogen. (——), intact PMV; (—o—), after removal of SDS-MCE by column chromatography on Sephadex G-50; (—x—), after G-25 concentration step.

microscopic examination of negatively stained preparations of TEV, PMV, and TEVI after removal of free SDS and subsequent concentration by G-25 Sephadex revealed amorphous material but not intact virus particles or inclusions.

All of the elongate viruses that were denatured with SDS-MCE were analyzed by liquid column chromatography using controlled pore glass column packing to assess the effectiveness of denaturation. Chromatograms were developed with SDS (0.1%) in 0.02 M sodium borate, pH 8.0, as the eluant. Monitoring and recording were as described in the section entitled Materials and Methods. Three peaks were resolved when the solutions of denatured viruses (PMV, PVX, CYMV, PeMV, PVY, and TEV) were chromatographed. The peaks were tested serologically and spectrophotometric data were collected. Figure 4 illustrates a typical chromatogram; CYMV was used as the example. Peak 2 was the only one found immunoreactive with CYMVSC antiserum in a gel immunodiffusion test. The spectrophotometric data indicated that the first peak was almost exclusively RNA (260/280 = 2.1) and the third peak possessed an absorption spectrum and odor typical of MCE.

SBMV preparations were denatured with SDS-MCE and analyzed by liquid column chromatography as described for the elongated viruses. The denatured SBMV preparations were resolved into three peaks (Figure 5). Fractions were collected and

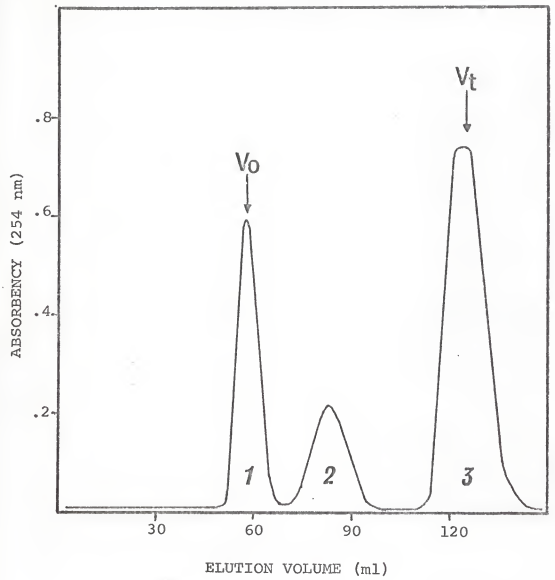


Figure 4. Typical elution pattern obtained when an SDS-denatured virus (CYMV) was chromatographed using CPG-370 A column packing and an SDS eluant (0.1% SDS in 0.02 M sodium borate, pH 8.0). (Vo), exclusion volume; (Vt), total inclusion volume; peak 1-RNA; peak 2-viral capsid protein; peak 3-MCE. The only immunoreactive peak in SDS-gel immunodiffusion using SDS-derived subunit antisera was Peak 2. Peak 1 typically possessed a 260/280 ratio of 2.1.

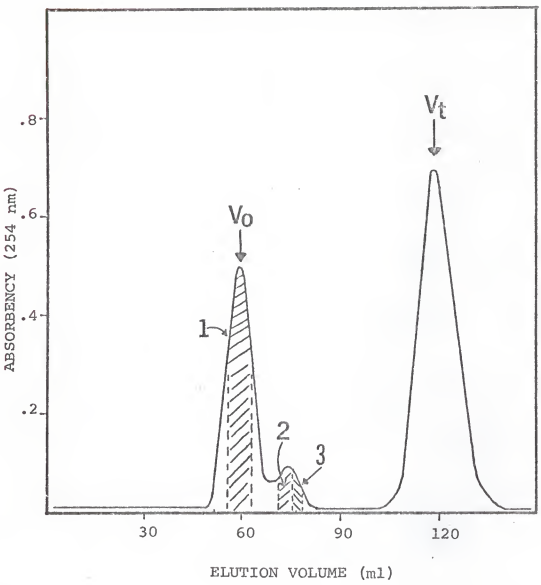


Figure 5. Elution pattern of SDS-denatured SBMV using CPG-370 A column packing. Numbered areas of peaks enclosed by dashed lines identify fractions collected and examined both serologically and spectrophotometrically. Only fractions 2 and 3 were immunoreactive with SBMVSC antiserum and none of the fractions were reactive with whole virus antiserum (SBMVI). Peak marked by Vt (total inclusion volume) was MCE and peak appearing at Vo was viral RNA. The eluant was 0.1% SDS in 0.02 M sodium borate, pH 8.0.

immunoreactivity of each fraction was assessed using antisera developed to the whole virus and to the SDS-denatured virus. These tests revealed that all fractions were devoid of immunoreactivity to the whole virus and only fractions two and three, corresponding to the elution position for subunit protein, were serologically reactive with the subunit antisera. The peak appearing at the V_t of the column was MCE. The 260/280 ratios of the fractions were as follows: 1, (2.13); 2, (0.64); and 3 (0.57).

SDS-polyacrylamide slab gel electrophoresis of formaldehyde-fixed, SDS-denatured PMV revealed the presence of several zones. Marker proteins (bovine serum albumin, glutamate dehydrogenase, tobacco mosaic virue, phosphorylase a, ovalbumin, alcohol dehydrogenase, and freshly SDS-denatured PMV) were simultaneously electrophoresed and their relative mobilities compared to that of formaldehyde-fixed PMV. Assuming a subunit molecular weight of 21,000 for PMV (Koenig et al., 1970), it appeared that upon removal of free SDS by column chromatography, the depolymerized capsid protein of PMV aggregated into multiples of the basic subunit. Based on the relative intensities of the stained zones, the dimeric aggregate predominated, and zones corresponding to monomeric and trimeric aggregates were also present.

Denaturation of PMV by Guanidine·HCl

PMV was readily denatured by guanidine·HCl. The ultraviolet absorption spectrum of the supernatant solution after removal of the precipitated RNA by centrifugation indicated that it was mostly protein. The 260/280 ratio was found to be 0.75, averaged from five experiments. Although guanidine·HCl was a good denaturant, several problems were encountered. First, when guanidine was removed by dialysis against an aqueous buffer, the protein precipitated from solution. The precipitated protein could be collected by centrifugation and exposed to 8 M urea, but much of it remained insoluble. Second, formaldehyde fixation of the protein could not be done in the presence of guanidine·HCl because formaldehyde and guanidine formed a spontaneous precipitate that also caused coprecipitation of much of the viral protein. Thus, in order to fix guanidine-denatured PMV with formaldehyde, while simultaneously maintaining protein solubility, it was necessary to perform the series of dialyses as described in the section on Materials and Methods. The ultraviolet absorption spectrum of the fixed protein was essentially unchanged from the spectrum for the high-speed supernatant solution.

Antigenic Specificity and Titers of Antisera

The antigenic specificity of PVYSC, TEVSC, PeMVSC, PeMVISC, TEVISC, and TEVNISC were examined by means of the SDS-gel immunodiffusion system. The antisera tested were the last collection dates titered as shown in Table 2. Tests also were conducted to determine if there was any antigenic relationship between the viruses and the viral-induced inclusions. Using purified preparations of TEV, TEVI, and TEVNI, it was clearly shown that the virus, virus-induced cytoplasmic inclusion, and the virus-induced nuclear inclusion were serologically distinct (Figure 6A). Similarly, PeMV and PeMVI were proved serologically unrelated. Other tests of the antigenic specificity of the various antisera were conducted with other PVY group viruses and virus isolates using lyophilized crude saps. The results are recorded in Table 1 and selected gel immunodiffusion patterns are shown in Figure 6 B, C, E.

Agar gel immunodiffusion tests were also conducted with pyrrolidine-denatured anigens of PeMV, PVY, and TEV derived from crude sap. The results were identical to those observed in the SDS-gel immunodiffusion system, except that TEVSC cross-reacted with the pyrrolidine-denatured PeMV antigen whereas it failed to do so in the SDS-gel medium.

Figure 6.

Agar gel immunodiffusion tests illustrating the various antigenic specificities of antisera elicited by SDS-denatured immunogens. Media were as follows: (A)-(E) and (G), 0.5% SDS; (F), borate buffered. The antisera in plates (A)-(G) were designated as follows: (a), TEVSC; (b), TEVNISC; (c), TEVISC; (d), PeMVISC; (e), PMVSC; (f), CYMVSC; (g), PVXSC. In (A), the specificity of TEVSC, TEVNISC, and TEVISC antisera was tested. Antigens were as follows: (1) and (3), excised zones from SDS-polyacrylamide gel electrophoresis of a purified preparation of TEVNI; (5), sample of the original purified preparation of TEVNI that was electrophoresed; (2), purified TEVI; (4) and (6), purified TEV. Note the lack of any cross reactivity between the three antigens. In plate (B) the antigens were SDS-resuspended, lyophilized crude sap extracts of the following: (1), healthy squash; (2), healthy tobacco; (3), healthy mustard; (4), lettuce mosaic virus-infected pea; (5), TEV-infected tobacco; (6), turnip mosaic virus-infected mustard; (7), bidens mottle virus-infected hybrid tobacco (Christie, 1969); (8), PeMV-infected tobacco; (9), PVY-infected tobacco; (10), pepper veinal mottle virus-infected tobacco; (11), tobacco vein mottle virus-infected tobacco; (12), watermelon mosaic virus-infected squash. Note the reactivity shown by the heterologous antigens (9), (10), and (11) and the spur formation with lettuce mosaic virus. In plate (C) the antigens were as follows: (1), PeMV-infected tobacco; (2) and (5), TEV-infected tobacco; (3), PVY-infected tobacco; (4), TEVs isolate from infected tobacco; (6), PVX-infected tobacco. The antigens were SDS-resuspended, lyophilized crude saps. Note the lack of cross reactivity of TEVSC (a) antiserum with PeMV antigen in contrast to that shown for the TEVISC (c) antiserum. In plate (E) the antigens were arranged as detailed for plate (B). In contrast to the TEVISC antiserum the PeMVISC antiserum did not possess any demonstrable heterologous reactivity in this or any other test. In plates (D), (F), and (G) the antigenic specificities of PVXSC, PMVSC, and CYMVSC antisera were tested. In (D), the antigens were SDS-resuspended, lyophilized crude saps of the following: (1), healthy pea; (2), CYMV-infected pea; (3) PMV-infected papaya; (4) healthy papaya; (5), healthy tobacco; (6), PVX-infected tobacco. In plates (F) and (G), the antigens were purified (1 mg/ml) virus. Plate (F) contains antigens denatured in 3% pyrrolidine and plate (G) contains antigens denatured in 1% SDS. The antigens were arranged as follows: (1) and (4), CYMV; (2), PVX; (3), PMV. Note the absence of any heterologous reactivity. Also note the difference between pyrrolidine-denatured CYMV versus SDS-denatured CYMV immunoprecipitates.


A
B
C
D
E
F
G

TABLE 1

Specificity of Antisera to Potato Y Group Antigens

Antigens	Antisera				
	TEVSC	PVYSC	PeMVSC	TEVISC	PeMVISC
TEV ATCC #PV-69	I [a]	O	O	I	O
PVY	S	I	S	S	O
PeMV	O	S	I	S	I
Bidens Mottle Virus	S	S	S	O	O
Turnip Mosaic Virus	O	O	S	O	O
Pepper Veinal Mottle	S	O	S	O	O
Lettuce Mosaic	S	O	O	O	O
Watermelon Mosaic II	O	O	O	O	O
Tobacco Vein Mottle	S	O	O	O	O
Wheat Streak Mosaic	O	O	O		
PVY #57[b]		I			
PVY #78[b]		I			
PVY #138[b]		I			
PVY FC-596[c]		I			
PVY FC-6[c]		I			
TEVs[d]	I	O	O	I	
PeMV FC-737[e]			I		I

[a] I - reaction of identity (no spur formation)
S - homologous reaction spurred over heterologous reaction
O - no reaction
Blank - not tested

[b] Gooding and Tolin, 1973

[c] PVY isolates from Florida grown tobacco

[d] an isolate from G. V. Gooding, Jr.

[e] an isolate from Florida grown bell pepper

TABLE 2

Titers of Antisera by Agar Gel Immunodiffusion

Antiserum &[a] Rabbit #	Date[b]	Date of Collection and Titer			
		Date[c]	Titer[d]	Date	Titer
TEVSC #779	29	37	4	116	8
TEVISC #786	28	34	2	89	4
TEVNISC #803	35	47	2	83	4
PVYSC #804	33	41	4	103	4
PeMVSC #783	29	39	4	86	8
PeMVISC #775	28	35	2	90	2
SBMVSC #806	30	40	4	88	4
PMVSI #776	42	50	2	84	2
PMVSC #769	23	42	4	78	4
PMVGI #755	42	50	4	78	4
PMVSCF #801	37	50	4	95	4
PVXSC #782	35	45	4	89	4
PVXSCF #792	41	52	4	90	4
CYMVSC #796	24	42	4	89	4
SBMVI #802	29	39	128	84	256
PMVI #747	23	30	1		

[a]the last one or two letters designate the following:
- S - depolymerized immunogen in SDS
- C - injection of immunogen emulsified in Freund's complete adjuvant
- I - injection of immunogen emulsified in Freund's incomplete adjuvant
- F - formaldehyde fixed immunogen
- G - depolymerized immunogen in guanidine·HCl

[b]days elapsed between the first and last injection

[c]number of days elapsed between first injection and date of collection of serum used for titering

[d]titer expressed as reciprocal of highest reactive dilution. All titers were determined by SDS-gel immunodiffusion except SBMVI #802, which was determined in borate immunodiffusion plates.

The antisera elicited by the SDS-denatured immunogens of PVX (PVXSC), CYMV (PMVSC), PMV (PMVSC) and SDS-denatured, formaldehyde-treated PMV (PMVSCF), and guanidine-denatured, formaldehyde-fixed PMV (PMVGI) were tested in a similar manner as described for SDS-derived subunit antisera of the PVY group. There were no antigenic relationships among either pyrrolidine-denatured or SDS-denatured antigens of PVX, PMV, and CYMV (Figure 6 D, F, G). The three PMV antisera (PMVSCF, PMVSC, and PMVGI) were also tested with formaldehyde-treated, SDS-treated, and guanidine-derived (formaldehyde-treated) PMV antigens in the SDS-gel immunodiffusion medium. All three antisera gave reactions of identity with the three types of antigens.

All the antisera were titered in the SDS-gel immuno-diffusion system except SBMVI, which was titered in the borate buffered medium. As shown in Table 2, most of the antisera had titers of 1:4 and only two antisera, TEVSC and PeMVSC, had titers of 1:8. It was noted that some antisera produced markedly more intense reactions at 1:4 than others, without visible reactivity at 1:8.

The titers of six antisera also were determined by means of pyrrolidine (3%) denaturation of purified antigens (1 mg/ml). The tests were conducted in the borate buffered immunodiffusion medium. The highest reactive dilutions for

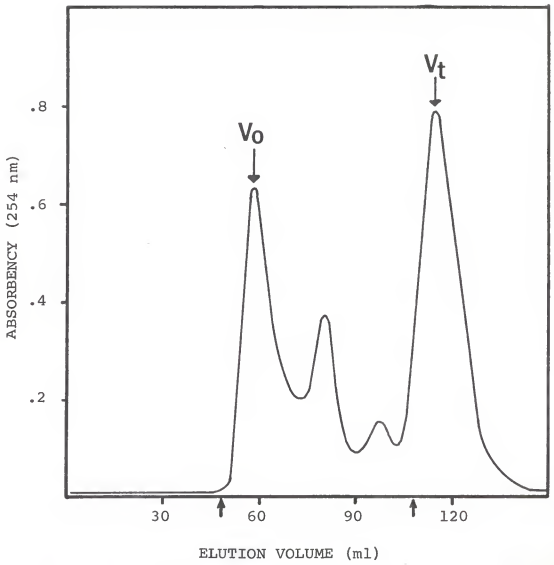
PMVSCF, PMVSC, PVXSCF, PVXSC, PeMVSC, and TEVSC were 1:8, 1:16, 1:32, 1:16, 1:32, and 1:64, respectively.

SDS-Column Chromatography for Purification of Inclusion Protein

To examine the feasibility of using liquid column chromatography for purification of inclusion protein, an impure inclusion preparation was denatured in SDS-MCE and chromatographed as described earlier. Fractions were collected in 5 ml samples and a small portion of each fraction was tested serologically for the presence of inclusion protein and virus coat protein using PeMVISC and PeMVSC subunit antisera, respectively. The remainder of each fraction was lyophilized, reconstituted to a volume of 1 ml, and retested. As shown in Figure 7, the inclusion preparation was resolved into several peaks. The immunoreactivity of the fractions (Figure 7) identified the peaks and revealed the presence of contaminating virus in the partially purified inclusion preparation. The first two 5 ml fractions collected were combined and lyophilized. When resuspended in 1 ml of deionized water, the material was rechromatographed (indicated by dash-dot line in Figure 8). The resulting peak was collected, concentrated to 1 ml by G-25 Sephadex, and tested in SDS-agar gel immunodiffusion using the viral (PeMVSC) and inclusion (PeMVISC)

Figure 7.

Chromatogram of a partially purified, SDS-denatured preparation of PeMVI and immunoreactivity of the collected fractions. Chromatography was performed using CPG-370 A column packing and an eluant of 0.1% SDS in 0.02 M sodium borate, pH 8.0. (Vo)-exclusion volume; (Vt)-total inclusion volume. Peak at (Vt) was MCE. Arrows at the abscissa indicate elution volume in which 5 ml fractions were collected. Immunodiffusion tests of collected fractions, as shown below the chromatogram, were performed in standard SDS-gel immunodiffusion medium. Center wells contain the antisera: (A) PeMVSC; (B) PeMVISC. Numbered peripheral wells contained the individual fractions with well 1 containing the first and well 11 the last fractions collected. The unmarked well contained only eluant as a control.


Vt
Vo
.8
.6
.4
.2
ABSORBENCY (254 nm)
30
60
90
120
ELUTION VOLUME (ml)


1
2
3
4
5
6
A
7
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4
5
6
B
7
8
9
10
11
B

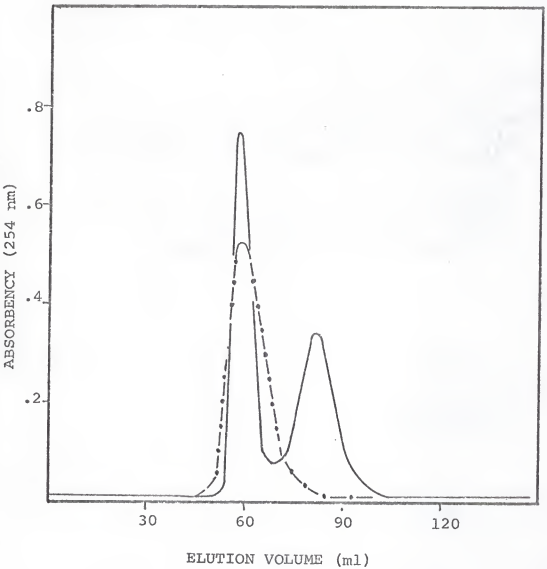


Figure 8. Composite of two chromatograms showing the elution of SDS-denatured PeMV (-—) and PeMVI (—•—) derived from the combined and concentrated first two 5 ml fractions obtained by chromatographing partially purified, SDS-denatured PeMVI (Figure 7). Column packing- CPG-370 A; eluant- 0.1% SDS in 0.02 M sodium borate, pH 8.0.

antisera. The antigen derived from this peak was strongly immunoreactive with the inclusion antiserum, but no immunoreactivity was detected with the virus antiserum. A sample of the concentrated peak was subjected by E. Hiebert to SDS-polyacrylamide slab gel electrophoresis (Hiebert and McDonald, 1973); its mobility was identical to that of unchromatographed PeMVI.

The Evaluation of Gel Immunodiffusion Systems Containing Either SDS or Other Reagents

The SDS-gel immunodiffusion system was examined to establish the optimum concentrations of SDS and sodium azide (SA) for incorporation into the medium. Immunodiffusion media thus contained either 0, 0.25, or 0.5% SDS and SA was used at a concentration of 1% in all media. In addition to testing various SDS concentrations, immunodiffusion plates were prepared using a constant SDS concentration (0.5%) and either 0, 0.25, 0.5, 0.75, or 1% SA. The test antigens were lyophilized, water-triturated, healthy and TEV-infected tobacco saps. These were resuspended in deionized water or 1% SDS. The antisera used were TEVSC, TEVISC, and normal serum. Results were recorded 24 hr after adding the reactants to the immunodiffusion plates.

In Figure 9, comparison was made between the three

concentrations of SDS incorporated into the agar. The effects of resuspending samples in water or SDS were also compared in the various media. Some of the results were as follows: (1) the absence of TEVI-antiTEVI immunoprecipitates when the crude sap was resuspended in water (Figure 9 B, D, F), (2) the formation of nonspecific precipitates when SDS was used to resuspend the crude saps but was not incorporated into the gel medium (Figure 9 A), (3) the increased immunoprecipitin intensity produced in the 0.25% SDS-medium as compared to that produced in the 0.5% SDS-medium, and (4) the increase in size of the nonspecific precipitin halos around antisera wells in the medium containing 0.25% SDS.

In Figure 10, the effects produced by decreases in the SA concentration incorporated into the media were compared. The water-and SDS-resuspended crude sap samples were also compared in the various media. Some of the results follow: (1) the gradual decrease in the reaction intensity of TEV-antiTEV and TEVI-antiTEVI as the SA concentration decreased (Figure 10, G, E, C, A), (2) the absence of TEVI-antiTEVI and substantially reduced, TEV-antiTEV immunoprecipitates when the antigen samples were resuspended in water (Figure 10 B), (3) absence of nonspecific precipitates (except halos around antisera wells), (4) the TEVI-antiTEVI reactions (Figure 10 D, F) could not be explained. It seems reasonable to assume

that these latter reactions were immunospecific however; the absence of a precipitin reaction in Figure 10 H raises some doubts as to their origin.

Finally, Figure 11 shows results of immunodiffusion in a medium that did not contain either SDS or SA. There were no reactions when water-resuspended crude sap was the antigen source, but reactions were obtained when SDS-resuspended crude sap was used. In the absence of incorporated SA, the SDS precipitated in the agar around the antigen wells. No explanation can be given for the absence of the SDS precipitate at the antigen well containing the infected crude sap. As seen before in Figure 9 A, the absence of SDS in the agar medium caused nonspecific precipitates to form in the antigen-antibody diffusion zone when SDS was present in the antigen sample. The presence of specific immunoprecipitates with both TEV and TEVI antisera was comparable to those formed in the medium containing only 1% SA (Figure 9 A). The intensity of these precipitates was greatest in the absence of incorporated SDS.

A study was made to determine if sodium chloride could be substituted for sodium azide in the standard SDS-gel immunodiffusion medium. Sodium chloride was used at a concentration (0.21 M = 1.25%) to provide an equivalent weight of sodium cation as that provided by 1% sodium azide. As can be seen

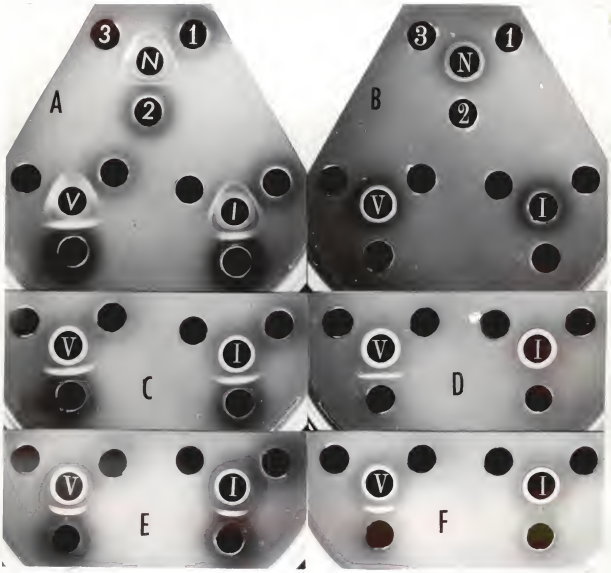
in Figure 11 C, the reactions in this medium were as good as those in the standard SDS-sodium azide-medium (Figure 9 E).

Tests were conducted to determine if the presence of urea in the agar gel medium would permit reduced concentrations of SDS to be used in the crude sap and/or the agar gel medium. Urea was incorporated into the immunodiffusion media alone and in combination with SDS and/or sodium borate buffer. The gels were prepared by dissolving 1.2% Noble agar in deionized water using a boiling water bath. After cooling the agar solution to 60 C, equal volumes of the following solutions at 60 C were added to equal volumes of the hot agar: 1 M urea in deionized water (urea alone); 1 M urea in 0.1 M sodium borate, pH 8.0 (urea + borate); 1 M urea and 0.4% SDS in deionized water (urea + SDS); 1 M urea and 0.4% SDS in 0.1 M sodium borate, pH 8.0 (urea + SDS + borate). The final concentrations of urea, SDS, agar, and buffer in the various media were 0.5 M, 0.2%, 0.6%, and 0.05 M, respectively. The test antigens were lyophilized, crude sap preparations from TEV-infected tobacco and were resuspended either in deionized water, 0.5% SDS, or 1% SDS. The antisera used were TEVSC, TEVISC, and normal serum. The immunodiffusion media were stored in a humid chamber at 4 C until used and results were recorded at 24 and 48 hr after the reactants were added to the plates.

The urea alone and urea + borate media produced similar

Figure 9.

Gel immunodiffusion tests comparing media containing 1% sodium azide and either 0, 0.25, or 0.5% SDS. Comparison also was made between the interrelationship of SDS concentration in the media to that used for resuspension of lyophilized crude sap antigens (0 or 1% SDS). Center wells contained antisera: (N) normal serum; (I) TEVISC; (V) TEVSC. Immunodiffusion media were as follows: (A) and (B) 0% SDS; (C) and (D) 0.25% SDS; (E) and (F) 0.5% SDS. Antigens in (A), (C), and (E) were resuspended in 1% SDS and were arranged in (A), (C), and (E) as shown for normal serum (N) in (A): (1) healthy tobacco sap; (2) TEV-diseased tobacco sap; (3) 1% SDS as control. Antigens in (B), (D), and (F) were arranged as in (A), (C), and (E) except crude saps were resuspended in water and (3) was water as a control. Note the absence of TEVI-antiTEVI reactivity in (B), (D), and (F) and the nonspecific precipitate formation in (A) when SDS was not present in the medium but was present in the antigen sample.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C
V
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D
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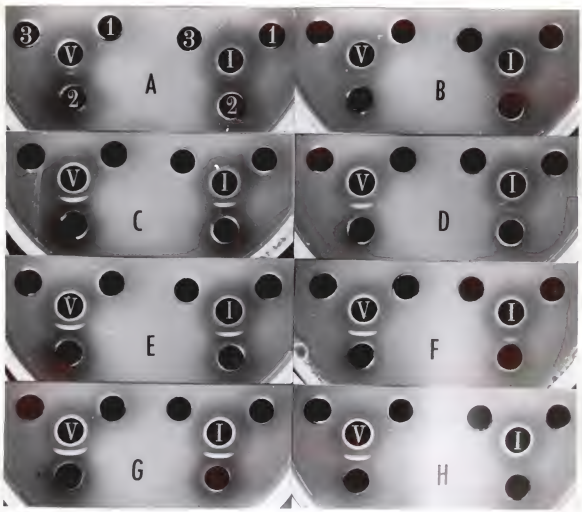
I
E
V
I
F

Figure 10.

Gel immunodiffusion tests comparing media containing 0.5% SDS and either 0, 0.25, 0.5, or 0.75% sodium azide. Comparison also was made between the interrelationship of sodium azide concentration in the media to the resuspension of lyophilized crude sap antigens either in 0 or 1% SDS. See Figure 9 for 1% sodium azide. Center wells contained the antisera: (V) TEVSC; (I) TEVISC. Normal serum controls were all negative and are not shown. The sodium azide concentration in the immunodiffusion media were as follows: (A) and (B) 0%; (C) and (D) 0.25%; (E) and (F) 0.5%; (G) and (H) 0.75%. Antigens in (A), (C), (E), and (G) were resuspended in 1% SDS and all were arranged as shown for plate (A) as follows: (1) healthy tobacco sap; (2) TEV-infected tobacco sap; (3) 1% SDS as control. Antigens in (B), (D), (F), and (H) were resuspended in water and arranged as shown for pattern (A) except well 3 contained water as a control. Note the increasing intensity of the TEV-antiTEV immunoprecipitates for both water resuspended and 1% SDS-resuspended crude sap as the sodium azide concentration increased. TEVI-antiTEVI immunoprecipitates also increased in intensity in the sodium azide series when 1% SDS-resuspended antigen was used. The reactions seen with TEVISC in (D), (F), and possibly (H) were of uncertain origin; although quite possibly these were indeed TEVI-antiTEVI immunoprecipitates their position differs from the inclusion reaction seen in (A) and the expected increase in reaction intensity fails to follow the pattern seen with the crude sap resuspended in 1% SDS. Finally, note the absence of nonspecific precipitates in all plates with the exception of halos around the antisera wells.


3
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V
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A
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V
1
B
V
1
C
V
1
D
V
1
E
V
1
F
V
1
G
V
1
H

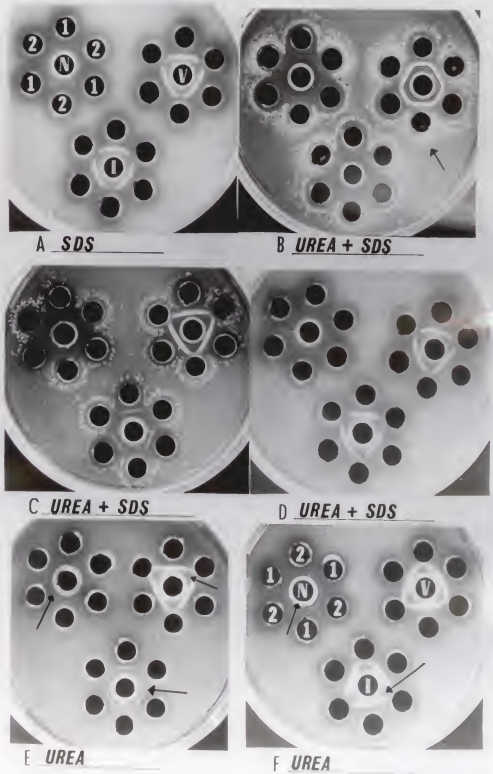
Figure 11.

Immunodiffusion in a medium devoid of both sodium azide and SDS. Medium was 0.6% Noble agar in deionized water. Center wells contained antisera and are designated as in Figure 10. Peripheral wells contained the resuspended crude sap antigens as follows: (1) healthy tobacco sap; (2) TEV-diseased tobacco sap. The immunodiffusion plate on the left (A) contained antigens resuspended in deionized water and well 3 was a deionized water control. The plate on the right (B) contained antigens resuspended in 1% SDS and well 3 was 1% SDS as a control. Note the total lack of any reactivity in (A). In Plate (B) note the granular precipitates at wells 1 and 3. This is assumed to be precipitated SDS. The precipitate was much reduced at well 1 and was absent at 2. The precipitate around the antisera wells closely resembles that observed in Figure 9 A (1% sodium azide was present in the gel phase but SDS was absent and the sample was resuspended in 1% SDS. Plate (C) was arranged as described for (A) and (B), however the medium was comprised of 1.25% sodium chloride and 0.5% SDS in 0.6% Noble agar (no sodium azide present). Note the resemblance of the immunoprecipitates in this medium compared to those in Figure 9 E.


1
N
I
3
2
V
1
N
I
3
2
V
1
N
I
3
2
V
C

Figure 12.

Tests of a urea containing gel immunodiffusion media. The types of media are listed below their respective immunodiffusion plates. Antisera were arranged the same for all media as shown in plate (A): (N) normal serum; (V) TEVSC; (I) TEVISC. Antigens were arranged in plates (A)-(E) as shown for normal serum (N) in (A) and arranged in plate (F) as shown for normal serum in (F): (1) TEV-diseased tobacco (2) healthy tobacco. The lyophilized crude saps were resuspended either in deionized water, 0.5% SDS, or 1% SDS and tested in the various media as follows: media (A), (D), (F) samples in 1% SDS; media (C) and (E) samples in 0.5% SDS; media (B) samples in water. Note nonspecific precipitates in media (B)-(F) as indicated by arrows and their absence, except for halos around antisera wells, in the SDS media (A). The nonspecific reaction with TEVSC (V) in (B) was absent in (C) and (D). Also note the progressive decrease in nonspecific precipitates through (B), (C), and (D) as the concentration of SDS in the sap increased from 0 to 1% and the concurrent increase in the TEVI-antiTEVI precipitin intensity.


1
2
2
N
1
1
2
V
I
A SDS
B UREA + SDS
C UREA + SDS
D UREA + SDS
E UREA
2
1
1
N
2
2
1
V
I
F UREA

results. Nonspecific precipitates occurred with all sera only when SDS was used to resuspend the lyophilized saps (Figure 12 E, F), and their intensities were essentially the same for 0.5% and 1% SDS-resuspended saps. Halos of non-specific precipitates were formed around all antisera wells when sap containing SDS was used. Water-resuspended samples neither produced specific nor nonspecific precipitates in either type of media. The formation of TEVI-antiTEVI immuno-precipitates occurred only when 1% SDS-resuspended sap was used (Figure 12 F). TEV-antiTEV reactivity occurred in both media when either 0.5% or 1% SDS was used to resuspend the crude sap antigens (Figure 12 E, F).

The urea + SDS and urea + SDS + borate media produced essentially the same results with the water, 0.5%, and 1% SDS-resuspended crude saps. The major difference was that with the urea + SDS medium, a nonspecific precipitate occurred in the agar beyond the circle of antigen wells with all sap treatments; however, the precipitates were substantially diminished when the crude saps were resuspended in 1% SDS (compare Figure 12, B-D). In both media, a precipitate formed with the TEVSC antiserum when water-resuspended samples were used (Figure 12 B). The precipitate did not occur either with TEVISC or normal serum and was not present in any other

media tested regardless of what was used to resuspend the lyophilized crude saps. TEVI-antiTEVI immunoprecipitates were present in both media when 0.5% SDS-resuspended crude sap was tested; however, their intensity increased dramatically when 1% SDS-resuspended sap was used (Figure 12 C, D).

In general, the urea + SDS and urea + SDS + borate media produced the most satisfactory results, but only if crude sap samples were resuspended in 1% SDS. There appeared to be no particular advantage in using any of these media instead of the SDS-gel immunodiffusion medium (Figure 12 A). Moreover, there is the uncertainty of reagent stability of the urea containing media under normal storage conditions (humid chamber at 4 C). For these experiments, the media were prepared and used within 24 hr; no study was undertaken to assess storage conditions. However, it was noted that during incubation at 25 C there was a slight odor of ammonia from the diffusion media, especially in the urea alone and urea + borate plates.

Additional agar gel immunodiffusion tests were performed to determine what influence various buffers incorporated into the agar would have upon the SDS-immunodiffusion system. Noble agar (0.6%) was dissolved in the various buffers, sodium azide (0.02%) was incorporated throughout and, in some media, 0.5% SDS was incorporated. Preparation of media was as

described in the section on Materials and Methods and the buffers were as follows: Tris (hydroxymethyl)-aminomethane (Tris), pH 8.1; sodium phosphate, pH 7.2; N-2-hydroxy ethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.5; and N-Tris (hydroxymethyl)-methylglycine (Tricine), pH 7.5; and sodium borate, pH 8.0. The test antigens were lyophilized, SDS (1%)-treated crude sap extracts from TEV-infected and healthy tobacco tissue; the antisera used were TEVSC, TEVISC, and normal serum. For controls, a medium of 0.6% Noble agar dissolved in deionized water with 0.02% sodium azide and the standard SDS-medium were used. In summary, the following media were prepared using two buffer concentrations of the five types of buffers listed above: 0.01 M buffer; 0.05 M buffer; 0.01 M buffer + SDS; 0.05 M buffer + SDS.

All the media composed of 0.01 M buffer without incorporated SDS formed intense, nonspecific precipitates. In the 0.05 M buffered media, without incorporated SDS, the strong, nonspecific precipitates were present, but the amount of precipitation was markedly reduced in the borate-buffered and Tricine-buffered media and specific lines could be discerned. Nonspecific precipitation also was a problem in the 0.01 M and 0.05 M media containing SDS. However, the nonspecific precipitates were confined to halos of varying intensity and diameters around the antisera wells. These

were most apparent in the media containing sodium phosphate, Tricine, and Hepes buffers. The media containing sodium borate and Tris buffers were essentially free of halo formation. In all cases where SDS was incorporated into the media, specific precipitates could be detected. In no case, however, were they as distinct or intense as those formed in the standard SDS-gel immunodiffusion medium. The medium composed only of agar and sodium azide contained nonspecific precipitates very similar to those observed in the 0.01 M buffered media.

The procedures used for preparing crude sap antigens for the SDS-gel immunodiffusion system were compared with regard to the intensity of reaction obtained and the ease of preparation and handling. The antisera used were TEVSC, TEVISC, and normal sera. TEV-infected tobacco leaves were harvested, cut into relatively uniform pieces, and two 5 g quantities were weighed. One 5 g batch was triturated in 10 ml of a 1% SDS solution and the other was triturated in 10 ml of deionized water. Both were divided into 3 aliquots. Two of the aliquots of each treatment were centrifuged for 30 min at 2600 g, the supernatant fractions were retained and the pellets from the water-triturated sap were resuspended either in 2 ml of deionized water or 2 ml of 1% SDS. The SDS-triturated sap did not form a pellet upon centrifugation.

The third aliquot from each treatment was used directly, without further treatment. The various samples were tested in the SDS-medium and were as follows: (1) SDS-triturated tissue; (2) water-triturated tissue; (3) water-triturated centrifuged sap pellet + SDS; (4) water-triturated centrifuged sap pellet + water; (5) water-triturated centrifuged sap supernatant solution. In addition, 1 ml quantities of the SDS- and water-triturated saps were lyophilized and were resuspended the next day either in 1 ml of water or 1 ml of 1% SDS, respectively. These resuspended saps also were tested by SDS-gel immunodiffusion using the same antisera as before. In all cases, healthy tobacco was processed in an identical manner as the TEV-diseased leaf tissue.

The sap from tissue which was triturated in water and added directly to diffusion wells produced a very weak inclusion reaction and a strong virus reaction. However, the supernatant from the water-triturated centrifuged sap failed to produce a detectable inclusion reaction and the virus reaction intensity was reduced. The pellets from the water-triturated centrifuged sap produced strong virus reactions whether resuspended in SDS or water. The inclusion reaction produced by the pellet resuspended in 1% SDS was much stronger than either of the water treatments (water-resuspended pellet or water-triturated sap used directly), and the inclusion

reaction produced by the water-resuspended pellet was stronger than the water-triturated sap used directly. The tissue triturated directly in SDS produced both a strong virus and inclusion reaction.

The lyophilized samples were tested after resuspension and compared with freshly prepared SDS-triturated crude sap. Both of the lyophilized preparations produced strong virus and inclusion reactions, comparable to those produced by the fresh sap preparation.

Immunoprecipitates formed in the SDS-gel immunodiffusion media usually were of the greatest intensity 24-36 hr after the addition of the reactants to the plate. However, after about 36-48 hr the precipitin lines begin to lose intensity, and in 4-5 days the precipitates often disappeared completely. This problem can be eliminated by adding a 10% slurry of activated charcoal (Norite) in deionized water to the emptied reactant wells at 24 hr diffusion time. Immunodiffusion plates so treated have been kept up to one year without noticeable loss of immunoprecipitin line intensity. In addition to the use of charcoal for preservation of precipitin lines, it may also be useful in the elimination of certain nonspecific precipitates. For instance, when the detergent N-lauryl sarcosine was used for denaturation of TEV, many nonspecific precipitates formed, making the test results inconclusive.

However, upon the addition of a 10% slurry of charcoal to the emptied depots, the nonspecific precipitates were eliminated within 24 hr, leaving only the specific precipitates (Figure 13).

Tests of Denaturants Other than SDS

To determine if chemicals, other than SDS, could produce antigenic fragments that would be soluble, diffusible, and immunoreactive with antibodies elicited by SDS-denatured immonogens, the following chemicals, at the indicated concentrations, were tested: 2-pyrrolidinone (3%), ethanolamine (3%), triethanolamine (3%), butylamine (3%), pyrrolidine (3%), pyridine (40%), formamide (30%), sodium formate (30%), N-lauryl sarcosine (1%), sodium dibutylnaphthalene-sulfonate (leonil, 1%). The antigens used were lyophilized crude saps of SBMV-infected bean, PMV-infected papaya, tobacco infected with TEV, and their respective healthy crude saps. Resuspension and immunodiffusion conditions were as described in the section on Materials and Methods. In addition to healthy sap as controls, antigen samples were resuspended in 1% SDS or deionized water.

The chemicals varied widely in their effects (Table 3). The only chemical that effectively depolymerized all the antigens tested was SDS (Figure 14, #s 1-6). TEV was degraded

Figure 13.

Gel immunodiffusion of N-lauryl sarcosine denatured antigens demonstrating removal of nonspecific precipitates by a slurry of decoloring charcoal. Antisera were as follows: (N) normal serum; (V) TEVSC; (I) TEVISC. Antigens were arranged in all patterns as shown for (N)) in the left plate and were as follows: (2) and (5) lyophilized healthy tobacco sap resuspended in 1% sarcosine; (3) and (6) TEV-infected, lyophilized crude sap resuspended in 1% sarcosine; (1) and (4) sarcosine (1% in water) as a control. Plate on the left was photographed 24 hr after the reactants were added and plate on the right was photographed at 48 hr. Antigen and antisera wells were cleaned of reactants at 24 hr and the depots were refilled with a slurry of 10% decolorizing charcoal in deionized water. Note the disappearance of the nonspecific precipitates in the plate at 48 hr and the remaining specific precipitates.


1
6
2
N
5
3
4
1
V
N
1
V

Figure 14.

Tests, by agar gel immunodiffusion, of the ability of various chemicals to disrupt TEV, TEVI, TEVNI, PMV, and SBMV into diffusible fragments serologically recognizable by antisera elicited by SDS-denatured immunogens. Antisera tested were as follows: (A) TEVSC; (B), TEVISC: (C), TEVNISC; (D), PMVGI; (E), PMVSC; (F), SBMVSC. The antigens were all lyophilized crude saps and were resuspended in the reagent listed immediately below the respective immunodiffusion patterns. The depot marked by an (R) contained the solution of the reagent used to resuspend the crude sap used in that particular pattern. The antigen arrangement in the peripheral depots is listed as follows according to which antiserum was in the center well: antisera (A), (B), and (C)- (1) healthy tobacco, (2) TEV diseased tobacco; antisera (D) and (E)- (1) healthy papaya, (2) PMV-infected papaya; antiserum (F)- (1) healthy bean, (2) SBMV-infected bean. Not the reactions obtained with the SDS-denatured antigens and the homologous antisera. The intensity of immunoprecipitates formed with pyrrolidine (8)- and ethanolamine (14)-denatured TEV were comparable to that in (1). However, contrast the TEVI-antiTEVI precipitates in (9) and (10) with that in (2). Note the SBMV-antiSBMV immunoreactuvity in (6) and (7). There were no other reagents tested, except SDS, that approached the intensity produced by pyridine-denatured SBMV. In (11) and (12), the arrows emphasize the precipitates noted in the text for SBMVSC, PMVSC, and PMVGI. Note the nonspecific precipitates in (11) and (13) with leonil and sarcosine (see Figure 13, for the use of charcoal to selectively remove nonspecific precipitates produced by sarcosine).

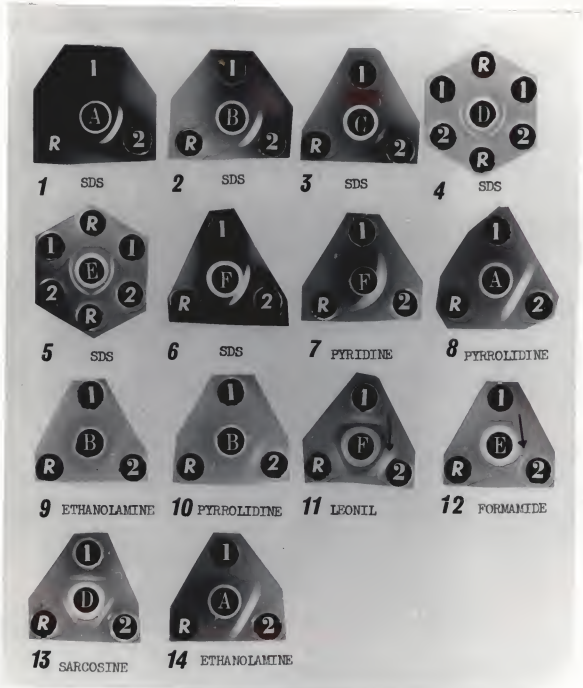

1 SDS
2 SDS
3 SDS
4 SDS
5 SDS
6 SDS
7 PYRIDINE
8 PYRROLIDINE
9 ETHANOLAMINE
10 PYRROLIDINE
11 LEONIL
12 FORMAMIDE
13 SARCOSINE
14 ETHANOLAMINE

TABLE 3

Ability of Various Chemicals to Disrupt TEV, TEVI, TEVNI, SBMV, and PMV into Soluble, Antigenic Fragments

Reagents and Concentrations	Antisera Tested					
	TEVSC	TEVISC	TEVNISC	PMVSC	PMVGI	SBMVSC
2-pyrrolidinone 3%	-	-	-	-	-	-
triethanolamine 3%	-	-	-	+	+	-
butylamine 3%	++++	-	-	-	-	+
sodium formate 30%	-	-	-	++++	++++	-
formamide 30%	++++	-	-	-	-	-
pyridine 40%	+	-	-	-	-	++++
pyrrolidine 3%	++++	+	-	++	+	++
ethanolamine 3%	++++	+	-	+	+	-
leonil 1%	-	-	-	-	-	-
sarcosine 1%	NS	NS	NS	NS	NS	NS
deionized water	-	-	-	+	+	-
SDS 1%	++++	++++	++++	++++	++++	++++

(+) = positive reaction with intensity indicated by number of plus signs (++++ = strongest reaction); direct comparisons apply only to the 12 treatments within a column.
(-) = no reaction
(NS)= unable to interpret due to nonspecific reactions.

Note: Ability of the chemicals to disrupt the antigens was based on intensity, position (close to antiserum well or about midway between wells), and shape (straight or arced around antiserum well) of precipitin lines formed in immunodiffusion tests with crude saps treated with the various chemicals. SDS-treated antigens tested in SDS-gel immunodiffusion medium; all others tested in the borate buffered system. Test antigens were lyophilized crude saps of the following: TEV-infected tobacco; PMV-infected papaya; SBMV-infected bean; healthy tissue for all of the above and the chemical used for resuspension were used as controls.

by pyrrolidine (Figure 14, #8) and pyrrolidine was moderately effective for PMV and SBMV. Ethanolamine degraded both TEV (Figure 14, #14) and PMV, but not SBMV. Although both pyrrolidine and ethanolamine denatured TEVI, the reactions were very weak and diffuse (Figure 14, #s 9 and 10). Other chemicals were either ineffective or were effective only for one or two antigens. For example, pyridine-denatured SBMV yielded an excellent reaction with SBMVSC antiserum (Figure 14, #7), but it was ineffective for all other antigens except TEV, in which case the immunoreactions were extremely weak and diffuse.

Of all the chemicals tested, leonil, sarcosine, and 2-pyrrolidinone were the least effective. Leonil and sarcosine tended to form large halos of nonspecific precipitates around the antisera wells. In addition, sarcosine generated many nonspecific precipitates which, without proper controls, easily could have been mistaken for specific immunoprecipitates (Figure 14, #13). In other tests with these detergents incorporated into the diffusion medium analogous to the SDS system, there were no obvious improvements in their performance. Leonil and sarcosine were tested at 0.5% and 1% in the agar. In addition, lyophilized sap samples resuspended in detergent (1% and 2%) or in water were tested. In no instance were satisfactory results obtained with the following crude sap derived antigens: PVY-infected tobacco, TEV-infected tobacco,

and papaya infected with PMV. The respective SDS-elicited subunit antiserum was used (PVYSC, TEVSC, and PMVSC).

Multiple precipitin lines were observed in some of the SBMV and PMV immunodiffusion plates. The tests involving PMV were conducted with PMVGI and PMVSC. The type of precipitin line marked by the arrow in Figure 14, #12 was observed with PMV-treated by all chemicals except SDS, pyridine, butylamine, and pyrrolidine. Furthermore, the precipitin line was not observed with normal serum, healthy crude sap, the chemical alone, or in tests in which purified, undenatured PMV was tested in the borate buffered gel immunodiffusion medium. A precipitin line, similar to that observed with PMV, was observed at the antigen well in tests of SBMVSC (Figure 14, #11). The line appeared in four treatments: 2-pyrrolidinone, sodium formate, leonil, and water. The reaction at the antigen depot was not present with normal serum, healthy bean crude sap, or the reagent alone; nor was it observed in tests with purified preparations of SBMV in borate buffered medium (no denaturant present). The precipitate at the diseased crude sap antigen well in tests involving both PMV and SBMV was unexpected. The obvious conclusion is that these subunit antisera (PMVSC, PMVGI, and SBMVSC) possess reactivity to the whole virus or at least reactivity to something other than the viral "subunit."

Depolymerization of PMV by Ethanolamine and Pyrrolidine

Column chromatography and agar gel immunodiffusion were performed to examine the depolymerization of PMV using either SDS, ethanolamine, or pyrrolidine. In addition, experiments were conducted to assess the relative diffusion rate in agar gel of pyrrolidine-denatured PMV and ethanolamine-denatured PMV.

Column chromatography was performed using the CPG-370 A column packing and eluants were as described earlier. The resulting chromatograms are shown in composite (Figure 15). Peak 1, which corresponds to the column Vo, absorbed strongly at 260 nm and exhibited a 260/280 of 2.1. It apparently consisted of viral RNA released by SDS denaturation. This peak was never observed when pyrrolidine-denatured PMV was chromatographed, regardless of the time of exposure of PMV to the denaturant. However, peak 1 was observed with ethanolamine-denatured PMV if the sample was chromatographed immediately after addition of the denaturant. The chromatogram shown in Figure 15 for ethanolamine-denatured PMV was obtained after 5 hr incubation prior to chromatographing the sample; note the absence of peak 1. The chromatogram obtained when the sample was chromatographed immediately after addition of ethanolamine was similar to that shown in Figure 15 for SDS-denatured PMV.

Effluent volumes corresponding to peaks 1, 2, 3, and 4 were collected, and tested in appropriate immunodiffusion media with PMVSC antiserum. All effluents were immunoreactive except for the one corresponding to peak 1. In addition, the precipitin lines fused (insert, Figure 15) when the ethanolamine and pyrrolidine effluents were tested in adjacent wells in an immunodiffusion plate.

Although the pyrrolidine and ethanolamine immunoprecipitates were serologically indistinguishable, the difference observed in their relative elution volumes suggested a definite difference either in size or shape. Therefore, tests were conducted to estimate the relative diffusion rates of the pyrrolidine- and ethanolamine-derived subunits by means of agar gel immunodiffusion. Allison and Humphrey (1959), developed a technique for estimating diffusion coefficients by double immunodiffusion in agar gel. In the test, the antigen and antibody diffuse at right angles to one another. If the test is conducted at optimal proportions (Aladjem et al., 1962), precipitates are formed along a plane which appears as a straight line. The right angle diffusion condition is established by means of a L-shaped trough. In the present study, the L-shaped pattern was formed by a template consisting of two stainless steel plates (40 x 2 x 15 mm) fixed by a support at right angles to one another. The two plates at

the point of intersection of the horizontal and vertical bars of the L were separated by 2 mm. The template was put in position in a petri plate and hot agar (0.6% Noble agar in 0.05 M borate, pH 8.0) was added to a depth of 4 mm; it was removed after solidification of the agar. One trough was filled with purified PMV denatured in either ethanolamine (3%) or pyrrolidine (3%) and the other trough was charged with PMVSC antiserum. Immunodiffusion plates were photographed 24, 48, and 72 hr after reactants were added to the troughs. Results were calculated using the following relationship which was modified from that given by Allison and Humphrey (1959):

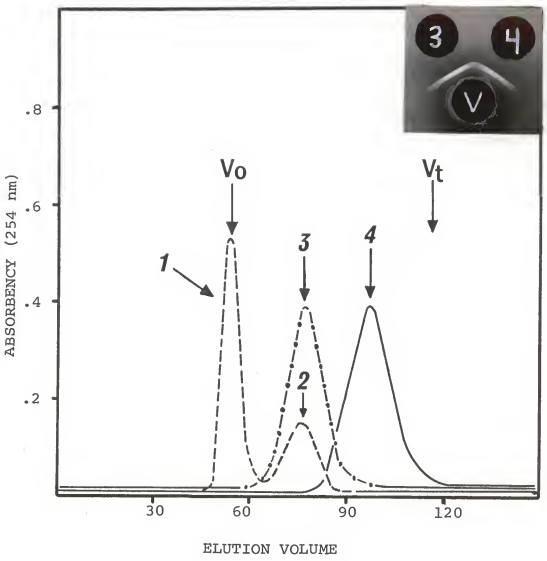
$$\frac{(\text{Tan } \theta_P)^2}{(\text{Tan } \theta_E)^2} = \frac{D_E}{D_P}$$

With reference to the above formula, θ was the angle of the precipitin line with the antigen trough, subscript (E) indicates ethanolamine, and subscript (P) indicates pyrrolidine. The relationship D_E/D_P represents the relative diffusion rate of ethanolamine-derived subunits to the pyrrolidine-derived subunits. Results indicated that pyrrolidine-derived subunits had a relative diffusion rate 1.2 times faster than the ethanolamine-derived subunits.

This was consistent with the elution behavior of pyrrolidine- versus ethanolamine-derived subunits in the column chromatography results (Figure 15).

Figure 15.

A composite of three different chromatograms in which purified PMV was denatured either by 3% ethanolamine, 3% SDS, or 3% pyrrolidine and then chromatographed in the respective eluant systems as follows: (-·-·-) 3% ethanolamine in deionized water; (- - -) 0.1% SDS in 0.02 M sodium borate, pH 8.0; (———) 3% pyrrolidine in deionized water. Column packing was CPG-370 A, (Vo) was exclusion volume, and (Vt) was the total inclusion volume. Peak 2, 3, and 4 were immunoreactive with PMVSC antiserum. Peak 1 was not immunoreactive and was composed of nucleic acid released by SDS denaturation of PMV. The immunodiffusion pattern shown as an insert at the top right of the chromatogram illustrates reactions obtained in borate immunodiffusion media using fractions from Peak 3 and 4. The numbered wells corresponded to the numbered peaks and (V) was PMVSC. Note the fusion of precipitin lines and the difference in the elution positions of the two antigens.


3
4
V
Vo
Vt
1
2
3
4
.8
.6
.4
.2
ABSORBENCY (254 nm)
30
60
90
120
ELUTION VOLUME

DISCUSSION

In this study, it was shown that SDS effectively denatured each of the viral capsid and inclusion proteins tested and that the denatured antigens were immunogenic. Moreover, of the chemicals tested, only SDS was capable of producing diffusible, immunoreactive antigens from each of the macromolecules studied. The SDS-agar gel immunodiffusion system, therefore, appears to possess a broad applicability for immunodiffusional analysis of virus-related antigens and for other protein antigens as well. Although some of the chemicals tested for their denaturing ability did produce diffusible, immunoreactive antigens, others did not. In these experiments, all serological tests were performed using antisera elicited by SDS-denatured immunogens. Thus, the possibility does exist that the chemicals that tested negative had indeed denatured the macromolecules but yielded fragments antigenically unrelated to those produced by SDS denaturation. On the other hand, PMVGI and PMVSC were shown to produce reactions of identity when tested against SDS-denatured PMV in the SDS medium and against guanidine-denatured formaldehyde-fixed PMV in the borate buffered medium.

Furthermore, Shepard et al. (1974) have demonstrated that SDS-denatured antigens of TEV and PVY were immunoreactive with antisera elicited by pyrrolidine-denatured TEV and PVY. Notwithstanding the preceding results, it is interesting to note the column chromatography and relative diffusion rate experiments in which ethanolamine-denatured PMV was compared to denaturation of PMV by pyrrolidine. The pyrrolidine-derived PMV subunits both eluted later and had a relatively faster diffusion rate than the ethanolamine-derived PMV subunits. In addition, the subunits produced by the two denaturants reacted identically in gel immunodiffusion tests with the PMVSC antiserum. It remains to be seen whether the behavior of pyrrolidine-derived subunits on the column and in the diffusion tests was due to a difference in hydrodynamic characteristics or to a truly different sized subunit from that produced by both SDS- and ethanolamine-denaturation.

The present and previous work suggests that the lamellar inclusion proteins and capsid proteins of the PVY group (Hiebert and McDonald, 1973), the capsid proteins of the PVX group (Koenig, 1972), and SBMV capsid protein (Hill and Shepherd, 1971) are denatured by SDS to give monomeric preparations. Denaturation of TEVNI by SDS however, apparently produces two electrophoretically distinct entities (Knuhtsen et al. 1974). Although most of the antigens were

depolymerized into monomers by SDS, evidence obtained by SDS-polyacrylamide gel electrophoresis of formaldehyde-fixed, SDS-denatured PMV indicated that, following column removal of free SDS, the monomeric subunits aggregated into multiple aggregation states with the dimeric form predominating. Consequently, the PMV immunogen consisted of subunit aggregates and not homogeneous monomeric units. In all probability, this also was the condition of the other immunogen preparations at the time of their injection. All antisera studied, however, reacted strongly with their respective SDS-denatured antigens and there was no indication that immunospecificity was influenced by the aggregated state of the injected materials.

The simplest procedure for antigen preparation from crude sap was direct trituration of the tissue samples in SDS. This procedure yielded excellent serological reactions both for the viruses and the inclusions. However, the centrifugation of water-triturated tissue sap may be advantageous for concentrating the inclusions and, thereby, increasing the sensitivity of the test. The centrifugation technique used also afforded some degree of purification. Techniques for handling fresh tissue can be more or less tailored to a particular need. In the present work, the use of lyophilized tissue sap was found to possess certain advantages over the use of fresh tissue. For example, lyophilized sap provided

a readily available source of standard antigen and partially obviated the need for continuous maintenance of a source of infected and healthy plant tissue in the greenhouse. In addition, no serological differences were found between the following: freshly prepared sap in SDS; SDS-triturated, lyophilized sap; and water-triturated, lyophilized sap re-suspended in SDS. In consideration of these points, it is suggested that water- or SDS-triturated, lyophilized sap from virus-infected plants could be utilized for interstate and international exchange of viral antigens. In certain instances this would eliminate the need for greenhouse propagation of potentially hazardous viruses, while permitting studies on serological relationships.

The SDS-gel immunodiffusion medium proved to be an effective system for conducting serological tests involving a wide variety of antigens. It was necessary that both SDS and sodium azide be incorporated into the medium and that SDS (1-1.5%) be present in the antigen sample for reliable detection of inclusion antigens (Figures 9-11). Although 0.5% SDS was used as a gel medium constituent throughout the present study, concentrations as low as 0.25% could be used. At reduced SDS concentrations, however, problems were sometimes encountered with nonspecific precipitates forming with certain sources of crude sap derived antigen (e.g., tomato,

datura). In fact, nonspecific precipitates were also formed in the 0.5% SDS gel medium, but their positions rarely interfered with or could be confused with the specific immunoprecipitates. In preliminary experiments, it was demonstrated that substitution of sodium chloride for sodium azide in the medium was entirely feasible and that the reactions obtained with the SDS-sodium chloride medium were as good as those obtained with the SDS-sodium azide medium. It appears that sodium chloride can be substituted for sodium azide. In addition, from experiments in which sodium azide concentration in the medium was varied, it was clearly shown that the immunoprecipitin intensities increased as the concentration of sodium azide increased (Figure 10). From these tests, it is postulated that the sodium cation in the presence of SDS, at the concentrations and temperature as described in this study, prevents significant alteration of the antibody - or, at least of its divalent combining sites. The literature discussed below appears to support this postulate.

SDS has been shown to be an effective inhibitor of the antigen-antibody precipitation reaction (Holmes, 1941; Kleinschmidt and Boyer, 1952). Cebra (1964) demonstrated reversible inhibition of antigen-antibody precipitation by SDS. In the presence of either 0.5% or 1% SDS, both intact immunoglobulin and an 85,000 molecular weight component

obtained by the consecutive action of papain and SDS were shown to be nonimmunoreactive. However, when the SDS concentration was reduced to 0.2% or less, immunological reactivity was restored. Cebra concluded that the antibody reactivity had been retained and that the intact immunoglobulin and the 85,000 molecular weight component were immunologically divalent. Although he did not study the interrelationships of ionic strength and SDS concentration upon the immunological properties of the immunoglobulin, he did employ a 0.1 M phosphate buffer, presumably sodium phosphate, for his studies. This would have provided an ionic strength in excess of that found in the SDS-sodium azide immunodiffusion medium used in this study. Furthermore, it has been shown that the ionic strength can significantly alter the amount of SDS bound to a protein. The ionic strength can thus alter the capacity of SDS to induce conformational transitions and can affect denaturation of the protein molecule into subunits, if it is composed of subunits. Reynolds and Tanford (1970a; 1970b) demonstrated that SDS in micellar form did not bind to proteins and that the effect of ionic strength was mainly upon the monomer-micellar equilibrium. Thus, the binding of SDS to proteins and the consequences of that binding depended upon the concentration of the SDS monomer present in solution. Similar results have been reported by others (Pitt-Rivers

and Impiombato, 1968; Nelson, 1971). Other properties of SDS-protein interactions have been investigated and the effects of ionic strength noted (Koshiyama, 1970; Lee and Jirgensons,1971). The effect of sodium chloride upon the antigen-antibody precipitin reaction has also been studied (Gill and Doty, 1961). It is not clear at this time if the ionic effect was due to the cooperative effect of all species in solution or only due to a certain one. In all cases cited however, and in the present work, the sodium ion was present in the solution by virtue of having been provided by the buffer, sodium chloride, or sodium azide. Nevertheless, evidence seems to implicate the sodium cation specifically as being responsible for inducing the effects observed.

Relating the above discussion to the present study, it can be suggested that most of the immunological and non-immunological reactions observed in the SDS-gel immuno-diffusion system were due to the interrelationship of the ionic strength and the SDS concentration in the medium. For example, Figure 11 B shows rings of granular precipitates around antigen wells 1 and 3, whereas these precipitates were absent in Figure 9 A. The only difference between the media was the incorporation of 1% sodium azide in the medium of Figure 9 A. Emerson and Holtzer (1967) showed that at constant temperature the critical micelle concentration

decreases as the ionic strength increases. By analogy, in the presence of 1% sodium azide (μ = 0.15 + contribution from agar medium) the critical micelle concentration was not met and, therefore, no precipitate formed. In the medium devoid of sodium azide, however, the critical micelle concentration was met at some point in the course of diffusion, and the precipitate did form. The marked reduction in precipitates and their absence at wells 1 and 2, respectively, in Figure 11 B, probably reflect a reduction in the amount of free SDS due to its binding to the plant sap constituents present in the crude sap samples. In contrast to the above, when SDS (0.5%) was present in the medium (Figure 10 A) and sodium azide was absent, not only were the granular precipitates about the antigen well absent but so also were the precipitin lines between the antisera and antigen wells. These precipitin lines were, in all probability, serum protein-SDS precipitates and reflect the parallel condition, as noted in this study, of SDS-denatured antigen precipitating from solution after removal of the free SDS by column chromatography. The precipitation of serum proteins by SDS has been reported by Neurath and Putnam (1945), Putnam and Neurath (1945), Jaquet et al. (1964), and Palmer et al. (1971).

In conclusion, the SDS-gel immunodiffusion medium was shown to be an effective system for conducting serological

tests involving both purified and crude sap-derived antigens (viruses and inclusions) when proper attention was given to the composition of the medium and in some cases, preparation of the antigen. It is believed that the analysis of the system given above explains failures cited for immunodiffusional analysis in SDS-containing gel medium, such as reported by Uyemoto et al. (1972). In addition, it was shown that sodium chloride could be substituted for sodium azide, thus eliminating a potentially dangerous chemical from the system.

Liquid column chromatography utilizing CPG column packing has been effectively used for the fractionation of a wide variety of substances such as bacteriophages (Gschwender et al., 1969), plant viruses (Marcinka, 1972), and immunoglobulins (Haller et al., 1970). In this study, advantage was taken of the chemical inertness, stable pore size and bed dimensions, and high flow rate characteristics of CPG. This column packing was used for fractionation of virus RNA and depolymerized capsid protein in an SDS-containing eluant, and the denaturation of PMV was examined using SDS, ethanolamine, and pyrrolidine as eluants. All these experiments were performed using the same column and column packing, only the eluant systems differed. The feasibility of purification of denatured inclusion protein by permeation chromatography using CPG was also demonstrated.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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